UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF PULLULANASE BY RAOULTELLA PLANTICOLA DSMZ 4617 USING SAGO STARCH AS CARBON SOURCE

HII SIEW LING.

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PRODUCTION OF PULLULANASE BY *RAOULTELLA PLANTICOLA* DSMZ 4617 USING SAGO STARCH AS CARBON SOURCE

By

HII SIEW LING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

March 2006

Especially dedicated to .....

Almighty God,

My dearest father, Hii Chee Ong and mother, Wong Pik Hah,

My brothers, Toh Ming and Toh Ping,

My sisters, Siew Chen and Siew Fei.

..... For all the nice things you've done : your patient, understanding, strong support, trust, thoughtfulness, guidance, love, care .....

AND for being there always in my times of need

THANKS A LOT !!!

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PRODUCTION OF PULLULANASE BY *RAOULTELLA PLANTICOLA* DSMZ 4617 USING SAGO STARCH AS CARBON SOURCE

By

HII SIEW LING

March 2006

Chairman : Professor Arbakariya Ariff, PhD

Institute : Bioscience

Production of pullulanase by *Raoultella planticola* DSMZ 4617 was studied in batch fermentation using sago starch as a carbon source. The fermentations were carried out, firstly, in 500-mL Erlenmeyer flasks to search for a suitable cultivation medium for pullulanase production by this gram negative bacterium. For comparison, production of pullulanase by a locally isolated strain was also carried out. Secondly, further improvement of pullulanase production by *R. planticola* DSMZ 4617 was done by using response surface methodology (RSM). The factors studied were sago starch, peptone and initial culture pH. The precise values of carbon-to-nitrogen (C/N) ratio that affect the pullulanase secretion by this gram-negative bacterium were also studied. Then, batch cultivations in 2-L stirred tank fermenter were carried out in an attempt to further improve pullulanase production by *R. planticola* DSMZ 4617. Two important hydrodynamic parameters, i.e., aeration and agitation, were studied in detail. Unstructured model based on logistic and Luedeking-Piret equations were

used to describe growth and pullulanase production by *R. planticola* DSMZ 4617 in both 500-mL shake flask and 2-L stirred tank fermenter.

Cultivation of *R. planticola* DSMZ 4617 in modified mineral Czapek medium was found able to produce substantially high activity of pullulanase (320 times higher) as compared to 'Ohba-Ueda' medium. Among various carbon and nitrogen sources tested, sago starch and peptone were the best substrates for enzyme production, and under these conditions, *R. planticola* DSMZ 4617 produced 0.95 U/mL of pullulanase at initial culture pH around 7 and incubation temperature of 30°C. The partially purified pullulanase from *R. planticola* DSMZ 4617 was optimally active at pH 6 to 7, and 50°C with stability ranges from pH 5 to 10. As compared with *R. planticola* DSMZ 4617, the local isolate *B. cereus* H1.5 was found to produce substantially high activity of protease during growth. This was the main reason that much of the pullulanase activity was lost during cultivation and partial purification processes and thus this local isolate is not appropriate for industrial applications. Therefore, the research work was focused on *R. planticola* DSMZ 4617.

The RSM experiments based on central composite design (CCD) were found practical to derive a statistical model for enhancement of pullulanase production by *R. planticola* DSMZ 4617. From this study, about 1.8-times of increment in pullulanase activity (1.70 U/mL) was achieved at 6.12 g/L sago starch, 15.34 g/L peptone and initial pH 7.23. Studies on C/N ratio further confirmed that the highest pullulanase production was obtained at ratio of 0.97 which correspondes to approximately 6.1 g/L of starch and 15.3 g/L peptone.

The pullulanase productivity and yield were greatly influenced by the aeration and agitation conditions within the fermenter. High pullulanase activities in the fermenter were observed at aeration rate of 0.5 vvm and agitation speed of 250 rpm. Under this condition, the pullulanase production results were: pullulanase activity, 2.22 U/mL; pullulanase productivity, 0.015 U/mL/h and pullulanase yield, 369 U pullulanase g starch^{-1}. The models proposed in this study fit significantly well to the experimental data with more than 95% confidence. This means that the proposed model can be used to explain growth and enzyme production at different chemical and physical conditions in a concise form which is comprehensible to those who wish to make use of the results. From the study, pullulanase production by *R planticola* DSMZ 4617 was found to be a non-growth associated process ($\alpha = 0$), where accumulation of pullulanase in the culture fluid occurred only during the non-growth phase.

An improved pullulanase fermentation process by *R. planticola* DSMZ 4617 has been successfully developed and it showed approximately 7-times increment of pullulanase production (2.22 U/mL) in 2-L stirred tank fermenter with optimized medium composition and culture conditions as compared to cultivation employing original medium without optimized formulation in 500-mL shake flask (0.32 U/mL).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN PULLULANASE OLEH *RAOULTELLA PLANTICOLA* DSMZ 4617 DENGAN MENGGUNAKAN KANJI SAGU SEBAGAI SUMBER KARBON

Oleh

HII SIEW LING

Mac 2006

Pengerusi : Profesor Arbakariya Ariff, PhD

Institut : Biosains

Penghasilan pullulanase oleh *Raoultella planticola* DSMZ 4617 telah dikaji secara fermentasi sesekelompok dengan menggunakan kanji sagu sebagai sumber karbon. Proses fermentasi telah dimulakan dengan menggunakan 500-mL kelalang kon di mana media pertumbuhan yang sesuai telah ditentukan untuk penghasilan pullulanase oleh bakteria gram-negatif ini. Secara perbandingan, penghasilan pullulanase oleh satu pencilan bakteria tempatan juga dijalankan. Kemudian, Kaedah Respons Permukaan (KRP) telah digunakan untuk meningkatkan lagi penghasilan pullulanase oleh *R. planticola* DSMZ 4617. Faktor-faktor yang dikaji ialah kanji sagu, pepton, pH permulaan kultur. Nilai tepat nisbah karbon-kepada-nitrogen (C/N) yang mempengaruhi perembesan pullulanase oleh bakteria gram-negatif ini juga dijalankan. Ini diikuti pula oleh pemeliharaan secara sesekelompok dalam fermenter berpengaduk 2-L yang bertujuan untuk meningkatkan lagi penghasilan pullulanase oleh *R. planticola* DSMZ 4617. Dua parameter hidrodinamik yang penting, iaitu, kadar alir udara dan kelajuan pengaduk telah dikaji

dengan teliti. Model tidak berstruktur berdasarkan persamaan logistik dan Luedeking-Piret telah dikaji untuk menerangkan pertumbuhan dan penghasilan pullulanase oleh *R. planticola* DSMZ 4617 dalam 500-mL kelalang kon dan fermenter berpengaduk 2-L.

Pertumbuhan *R. planticola* DSMZ 4617 dalam media galian Czapek yang diubahsuai berupaya menghasilkan amaun enzim pullulanase yang lebih banyak (320 kali lebih tinggi) kalau dibandingkan dengan menggunakan 'Ohba-Ueda' media. Antara pelbagai sumber karbon dan nitrogen yang dikaji, kanji sagu dan pepton didapati adalah substrat yang terbaik untuk penghasilan enzim dan di bawah keadaan ini, *R. planticola* DSMZ 4617 menghasilkan 0.95 U/mL pullulanase pada pH 7 dan suhu pengeraman 30°C. Pullulanase yang ditulenkan separa adalah paling aktif antara pH 6 dan 7 pada 50°C dengan kestabilan dari pH 5 ke 10. Jika dibandingkan dengan *R. planticola* DSMZ 4617, pencilan bakteria tempatan *B. cereus* H1.5 didapati menghasilkan aktiviti protease yang sangat tinggi semasa pertumbuhannya. Ini merupakan sebab utama aktiviti pullulanase merosot semasa proses pertumbuhan dan penulenan separa dan oleh itu, pencilan bakteria tempatan ini tidak sesuai digunakan untuk aplikasi industri. Oleh itu, eksperimen ini difokuskan dengan menggunakan *R. planticola* DSMZ 4617.

Eksperimen KRP berdasarkan Rekabentuk Komposit Tengah (CCD) didapati sangat sesuai unutk menghasilkan satu model statistik untuk penambahan penghasilan pullulanase dari *R. planticola* DSMZ 4617. Dari eksperimen ini, kira-kira 1.8 kali penambahan aktiviti pullulanase (1.70 U/mL) telah dicapai dengan menggunakan 6.12 g/L kanji sagu, 15.34 g/L pepton dan pada pH permulaan 7.23. Kajian nisbah

C/N membuktikan lagi bahawa penghasilan pullulanase adalah paling berkesan dengan nisbah 0.97 yang memerlukan 6.1 g/L kanji sagu dan 15.3 g/L pepton.

Produktiviti dan penghasilan pullulanase amat dipengaruhi oleh kadar alir udara dan keadaan pengadukan dalam fermenter. Aktiviti pullulanase yang tinggi dalam fermenter telah dicapai pada kadar alir udara 0.5 vvm dan kelajuan pengaduk 250 rpm. Di bawah keadaan ini, keadaan perembesan pullulanase adalah: aktiviti pullulanase, 2.22 U/mL; produktiviti pullulanase, 0.015 U/mL/j dan penghasilan pullulanase sebanyak 369 U pullulanase g kanji^{-1}. Model yang dicadangkan dalam eksperimen ini didapati berpadanan kepada data eksperimen dengan keyakinan melebihi 95%. Ini bermakna model yang dicadangkan boleh digunakan untuk menerangkan pertumbuhan dan penghasilan enzim secara ringkas dan padat untuk keadaan kimia dan fizik yang berlainan. Dari kajian permodelan ini, didapati penghasilan enzim pullulanase oleh *R. planticola* DSMZ 4617 merupakan proses pertumbuhan tidak berkait ($\alpha = 0$), di mana pengumpulan enzim pullulanase di dalam cecair kultur hanya berlaku pada fasa tanpa pertumbuhan.

Dari kajian ini, satu proses fermentasi pullulanase yang diperbaiki telah berjaya dibangunkan untuk *R. planticola* DSMZ 4617 dan kira-kira 7 kali penambahan penghasilan pullulanase (2.22 U/mL) dalam fermenter berpengaduk 2-L yang menggunakan komposisi media dan keadaan kultur yang maksima berbanding dengan menggunakan media asal tanpa formula optima dalam 500-mL kelalang kon (0.32 U/mL).

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THANK YOU

I certify that an Examination Committee has met on 20th March 2006 to conduct the final examination of Hii Siew Ling on her Doctor of Philosophy thesis entitled "Production of Pullulanase by *Raoultella planticola* DSMZ 4617 Using Sago Starch as Carbon Source" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Foo Hooi Ling, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Suraini Abdul Aziz, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Lai Oi Ming, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Abdul Jalil Abdul Kader, PhD
Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

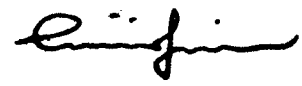
Date: 26 APR 2006

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Arbakariya Ariff, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Ling Tau Chuan, PhD
Lecturer
Faculty of Engineering
Universiti Putra Malaysia
(Member)

Rosfarizan Mohamad, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)



AINI IDERIS, PhD
Professor / Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 MAY 2006

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at Universiti Putra Malaysia or other institutions.

HII SIEW LING

Date: 24 Apr 2006

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g	Acceleration of gravity (~9.8 m/s/s)
α-D-glucopyranose	Alpha-D-glucopyranose
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
C	Carbon
C/N ratio	Carbon-to-nitrogen ratio
Co^{2+}	Cobalt ion
CFU	Colony forming units
CD	Cyclodextrin
DP	Degree of Polymerization
DEAE-cellulose	Diethylaminoathyl-cellulose
DOT	Dissolved oxygen tension
EC	Enzyme commission number
Fe^{2+}	Ferrous ion
g	Gram
Glu-1-P	Glucose-1-phosphate
h	Hour
H	Hydrogen
HCl	Hydrochloric acid
kDa	Kilodalton
L	Liter
λ_{max}	Maximum wavelength in nanometer